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14. ABSTRACT The insulin-like growth factor (IGF) pathway plays an important role in breast cancer growth and metastasis. The IGF-I receptor (IGF-IR) is over-expressed in almost 50% of triple negative breast cancers (TNBC) and is associated with poor prognosis and drug resistance. Thus, therapeutically targeting tumor cells which have upregulated IGF-IR may be a promising approach to treat TNBC. We report that IGF-IR is immunogenic. Through vaccination, high levels of IGF-IR Th1 could be generated which elicited IFN-g-dependent breast cancer inhibition. SOCS1, upregulated by IFN-g, bound IGF-IR. This interaction inhibited receptor signaling, modulated additional oncogenic proteins, and increased PTEN expression. Oncogenic shock, induced by immunization, restored sensitivity to Tamoxifen therapy in mice refractory to treatment. Cytokine mediated oncogenic shock may be a mechanism by which cancer vaccines, or other immunotherapies, improve response to subsequent standard treatments resulting in a survival benefit in cancer patients treated with immune modulatory approaches.				
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INTRODUCTION

The insulin-like growth factor (IGF) pathway plays an important role in breast cancer growth and metastasis. The IGF-I receptor (IGF-IR) is overexpressed in almost 50% of triple negative breast cancers (TNBC), defined as estrogen (ER) and progesterone (PR) receptor and HER-2/neu receptor (HER2) negative. We have determined that IGF-IR is immunogenic in breast cancer and is a potential target for active immunization.

Overexpressed growth factor receptor proteins, including IGF-IR, have been identified as addiction oncogenes in breast cancer as well as other tumors.^{1,2} Tumor cells that have become dependent upon a single activated oncogene for their growth and survival are thought to become oncogene addicted³. Investigations suggest that oncogene addiction imbalances cell senescent pathways in favor of anti-apoptotic signaling.¹ When a dominant oncogene is acutely inactivated, however, the balance is reversed in favor of pro-apoptotic signaling. The resultant oncogenic shock provides a window of enhanced sensitivity to cell killing prior to addiction developing to other growth or survival signaling pathways.^{4,5}

We hypothesize that immunologic targeting of overexpressed “biologic driver” proteins, those present in oncogenic pathways to which the tumor is addicted,¹ may impart clinical benefit even if induced antigen specific immunity does not completely eradicate the disease. Immune mediated elimination of malignant clones, which have upregulated proteins that confer a growth advantage to the tumor, may result in the development of a cancer remodeled to become a less aggressive variant of the original disease.

The specific aims of this proposal are to: (1) To identify putative Class II epitopes, derived from IGF-IR, that stimulate IGF-IR-specific T cells in patients with breast cancer; (2) To evaluate the immunogenicity, clinical efficacy, and safety of an IGF-IR class II polyepitope vaccine in a mouse model of TNBC.

BODY

Aim 1. To identify putative Class II epitopes, derived from IGF-IR, that stimulate IGF-IR-specific T cells in patients with breast cancer.

Aim 1.a. To identify IGF-IR peptides based on predicted high avidity binding across multiple class II alleles.

We have identified that 19/20 IGF-IR peptides predicted to bind with high affinity to multiple MHC class II alleles are immunogenic. Furthermore, we determined that there was significant IFN- γ response to peptides in the C-terminal domain (CTD) compared to the extracellular domain (ECD) or the kinase domain (KD), as discussed in the previous report. Work on this sub-aim is complete.

Aim 1.b. To determine whether IGF-IR-peptide specific T cell lines can recognize human recombinant IGF-IR protein presented endogenously by autologous antigen presenting cells (APC).

All the IGF-IR predicted peptides were native MHCII epitopes and the T cell lines generated using these peptides secreted predominantly Type 1 cytokines as described in the previous report. Work on this sub-aim is complete.

Aim 1.c. To determine whether identified IGF-IR peptides stimulate T regulatory (Treg) cell proliferation.

We determined that there was significant IL-10 response to peptides in the ECD or KD, compared to the CTD. We picked four peptides to include in our final vaccine formulation (IGF-IR-CTD peptide pool: p1301-1316, p1307-1321, p1212-1226 and p1166-1180) based on the magnitude and incidence of the IFN- γ and IL-10 response as discussed in the previous report. Work on this sub-aim is complete.

Aim 2. To evaluate the immunogenicity, clinical efficacy, and safety of an IGF-IR class II polyepitope vaccine in a mouse model of TNBC.

Aim 2.a. To determine the immunogenicity and therapeutic efficacy of IGF-IR immunization.

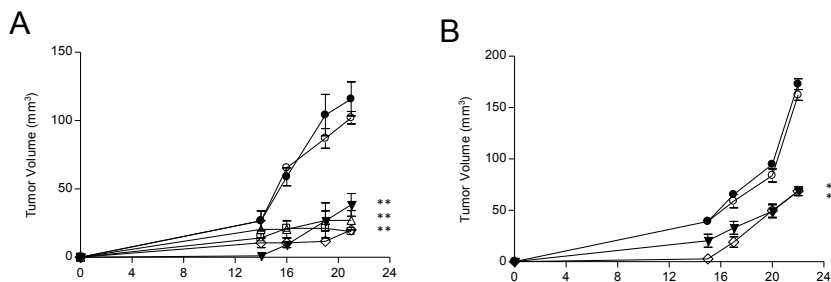


Figure 1. Th1 cells, elicited by IGF-IR vaccination, inhibit the growth of breast cancer via IFN-g secretion. (A) Mean tumor volume ($\text{mm}^3 \pm \text{SEM}$) from mice injected with adjuvant (●) IGF-IR vaccine (◇), or IGF-IR vaccine with isotype control Ig (▼), CD8⁺ depletion (Δ) CD4⁺ depletion (○) or B cell depletion (□). (B) Mean tumor volume ($\text{mm}^3 \pm \text{SEM}$) after mice were injected with adjuvant (●) IGF-IR vaccine (◇), or IGF-IR vaccine with isotype control Ig (▼) or IFN-g depletion (○); ** $p < 0.001$ for all panels.

IGF-IR-CTD peptide pool immunization generated a significant Th1 immune response and inhibited tumor growth in the triple negative breast cancer mouse model, TgC3(l)-Tag. Work on this sub-aim is complete.

Aim 2.b. To determine which immune effector arm is essential for mediating therapeutic efficacy after immunization.

To assess which T-cell subset mediated the antitumor effect, IGF-IR vaccinated mice were selectively depleted of CD4⁺ and CD8⁺ T-cells prior to tumor challenge. B-cells were also depleted as IGF-IR specific antibodies have been shown to mediate tumor regression.⁶ IGF-IR vaccination with adjuvant induced similar tumor inhibition as vaccination with CD8⁺ ($p = 0.376$) or B-cell depletion ($p = 0.75$) or isotype control Ig (clg) treatment ($p = 0.546$). However, depletion of CD4⁺ T-cells abrogated the anti-tumor effect of IGF-IR vaccination and tumor growth was no different from the adjuvant only controls, $p = 0.339$ (Fig. 1A).

As the predominant cytokine secreted by the IGF-IR specific Th cells was IFN-g, we selectively depleted IFN-g in vaccinated mice prior to tumor challenge to determine its contribution to the observed anti-tumor activity. The average tumor volume in IFN-g neutralized mice was significantly larger than untreated vaccinated mice ($p < 0.0001$), or clg treated vaccinated mice ($p < 0.0001$) (Fig. 1B). Indeed, tumor size in the vaccinated IFN-g-depleted animals was equivalent to adjuvant only controls ($p = 0.207$).

Tumor proliferation was significantly decreased in the IGF-IR immunized animals compared to the adjuvant controls, $p < 0.001$ (Fig. 2A and B). However, after IFN-g depletion, the percentage of proliferating tumor cells in vaccinated mice was not significantly different from the control ($p = 0.108$) (Fig. 2A), and was greater than that observed in tumors from vaccinated ($p < 0.001$) and clg treated vaccinated mice ($p = 0.008$) (Fig. 2A; representative fields Fig. 2B (I-IV)). In addition, apoptosis was significantly increased in tumors from both IGF-IR vaccinated and clg treated vaccinated mice compared to adjuvant only and IFN-g depleted vaccinated mice ($p = 0.002$, $p = 0.01$, respectively) (Fig. 2C and D). There was no difference in apoptosis in IFN-g

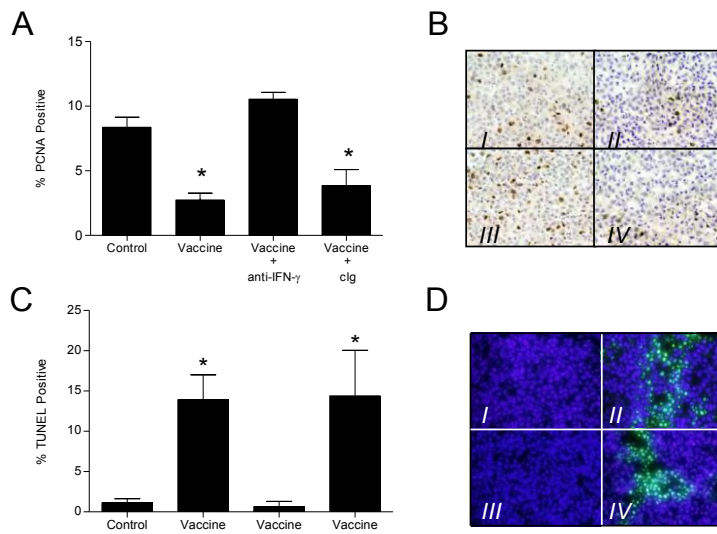


Figure 2. IFN-g modulates tumor cell growth and survival. (A) Percent PCNA positive cells $\pm \text{SEM}$, in 21 day tumors, were counted from 10 high-powered fields (HPF) from 6 mice; * $p < 0.01$. (B) Representative PCNA stain. (C) Percent TUNEL positive cells $\pm \text{SEM}$, in 21 day tumors, were counted from 10 high-powered fields (HPF) from 6 mice; * $p < 0.01$. (D) Representative TUNEL stain; I: control; II: IGF-IR vaccine; III: vaccine + anti-IFN-g; IV: vaccine + clg.

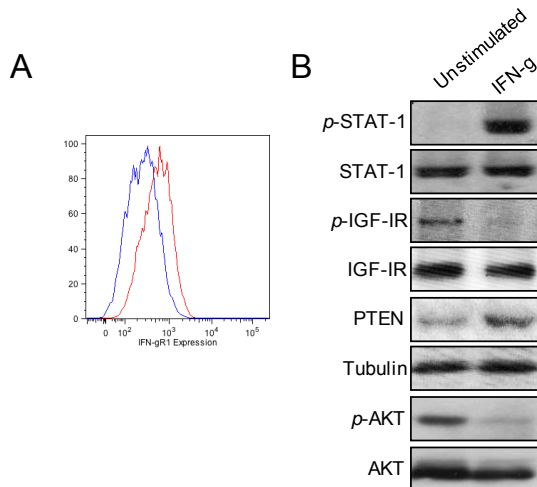


Figure 3. IGF-IR signaling is modulated by IFN-g. (A) Tumor cells were analyzed by flow cytometry; IgG isotype control (blue line), IFN-gR1 (red line). (B) Western blot of signaling proteins and tubulin.

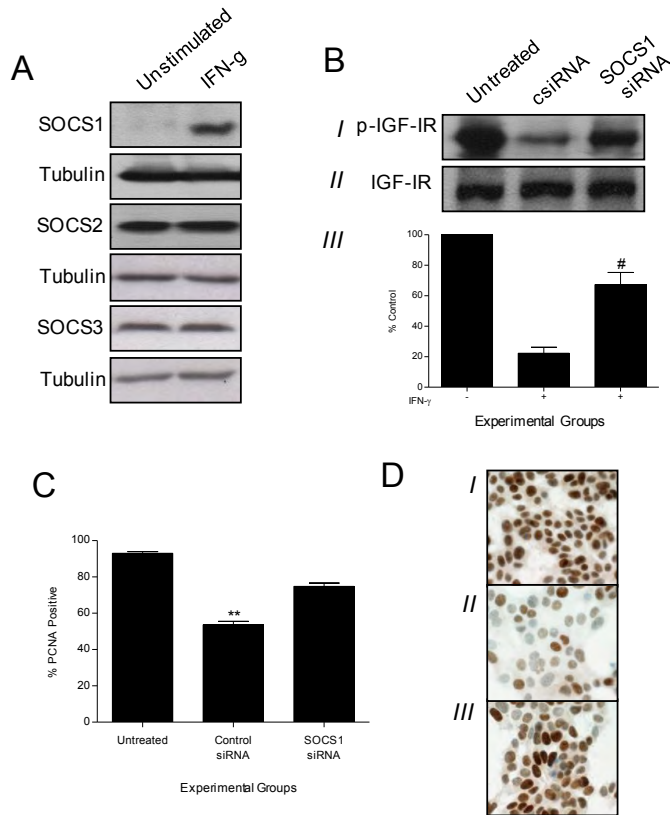


Figure 4. IFN-g-induced SOCS1 mediates IGF-IR activity. (A) Western blot of SOCS family proteins, unstimulated and after IFN-g treatment. (B) Representative Western blot of untreated and IFN-g + control (c) siRNA and SOCS1 siRNA transfected MMC for phospho- (I) and total IGF-IR (II). Percent control of untreated cells±SEM as measured by densitometry for csiRNA and SOCS1 siRNA (III); #p<0.01 compared to csiRNA + IFN-g treated cells (n=3 independent experiments). (C) Percent PCNA positive cells±SEM were counted from 10 HPF from 3 independent experiments for untreated and IFN-g treated csiRNA and SOCS1 siRNA transfected tumor cells, **p<0.001. (D) Representative PCNA stain; I: untreated; II: csiRNA + IFN-g; III: SOCS1 siRNA + IFN-g.

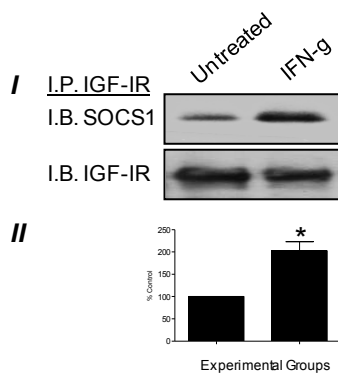


Figure 5. IFN-g potentiates the association between SOCS1 and IGF-IR. Representative Western blot of co-immunoprecipitation of SOCS1 and IGF-IR from tumor cell lysate (I); I.P.: immunoprecipitation, I.B.: immunoblot. Densitometry is represented as percent control of untreated cells ± SEM from three independent experiments (II); *p<0.01.

depleted vaccinated mice and adjuvant control tumors (p=0.575) (Fig. 2 C and D).

IFN-g receptors are expressed on human breast cancer cells^{7,8} and we similarly demonstrate that IFN-g-R1 is expressed on a syngeneic murine tumor cell line (Fig. 3A). *in vitro* IFN-g treatment of human breast cancer cells has been shown to restore STAT1 signaling,⁹ as it did in tumor cells (Fig. 3B). Figure 3B also demonstrates IFN-g treatment of tumor cells inhibits signaling through IGF-IR without significantly altering receptor protein levels. As enhanced IGF-IR signaling has been shown to be associated with a loss of PTEN protein expression in breast cancer, conferring a growth advantage to PTEN negative cells,¹⁰ we assessed PTEN restoration with IFN-g treatment. The level of PTEN protein was increased after IFN-g exposure (Fig. 3B). As PTEN negatively regulates the PI3K/AKT pathway,¹¹ we evaluated phospho-AKT expression after IFN-g treatment, and demonstrated a marked decrease in activity (Fig. 3B).

We questioned whether SOCS family members were operative in modulating IGF-IR signaling as SOCS proteins have been shown to attenuate signaling through insulin receptor, which is highly homologous.¹² Compared to SOCS2 and 3, SOCS1 expression in tumor cells was strongly induced with IFN-g treatment (Fig. 4A). To determine whether SOCS1 was regulating IGF-IR phosphorylation in the presence of IFN-g, we silenced its expression. Untreated MMC cells demonstrate high levels of IGF-IR phosphorylation which was significantly inhibited (p=0.019) with IFN-g treatment in siRNA control cells (csiRNA) (Fig. 4B I,III). Reduction of SOCS1 levels via siRNA, in the presence of IFN-g, resulted in 67%

restoration of IGF-IR signaling, compared to IFN-g csiRNA treated cells (p=0.007) (Fig. 4B I,III). IFN-g inhibited proliferation of tumor cells treated with csiRNA compared to untreated cells (p<0.0001) (Fig. 3f). Decrease in SOCS1 expression, in the presence of IFN-g did not significantly restore tumor proliferation to the level of the untreated control (p<0.001) but it did increase cell proliferation by 26% compared to csiRNA (p<0.0001) (Fig. 4C; representative fields Fig. 4D I-III).

Investigators have hypothesized that SOCS1 may directly interact with insulin receptor to inhibit activation of its downstream effectors¹³ and we questioned whether SOCS1 may be directly binding to IGF-IR in our model. Figure 5 I demonstrates significant co-precipitation of SOCS1 with IGF-IR from IFN-g-treated tumor cells compared to controls (p=0.007, Fig. 5 II).

Since overexpression of AKT induces Tamoxifen resistance, while inhibition restores Tamoxifen sensitivity,¹⁴ we questioned whether our IGF-IR-specific vaccine would render previously Tamoxifen-resistant tumors now sensitive. Vaccination demonstrated an inhibition of tumor growth by 85% compared to adjuvant only control animals (p<0.001) (Fig.6). Tumor growth in mice injected with adjuvant and treated with Tamoxifen was no different than that

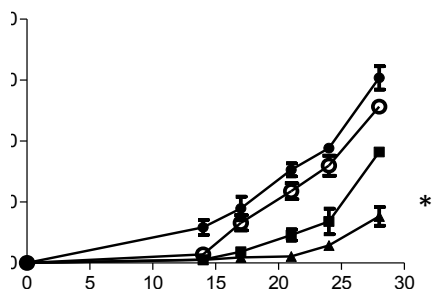


Figure 6. IGF-IR-specific vaccine sensitizes tumors to Tamoxifen therapy. Mean tumor volume (\pm SEM) of mice injected with adjuvant (●), IGF-IR vaccine (■), or treated with Tamoxifen after injection of adjuvant (○) or IGF-IR vaccine (▲). * $p < 0.001$; $n = 5$ mice / group.

development every two weeks where I have presented data as well as given introductory lectures on new concepts within my scope of work that was unknown to the group.

I have attended and presented my data at the annual American Association of Cancer Research meeting. I have participated in many seminars and short courses offered by the University of Washington's Institute for Translational Health Sciences on career development and clinical education. I have also witnessed the consent process involved in enrolling a patient on a clinical trial.

I have worked closely with my mentor on submitting a pilot grant and the preparation of two manuscripts: "Adiposity is associated with enhanced Th1 immunity to the Insulin-like Growth Factor Receptor" and "Interferon- γ mediated oncogenic shock sensitizes breast cancer to biologic therapy after vaccination targeting IGF-IR."

KEY RESEARCH ACCOMPLISHMENTS

- Th1 cells, elicited by IGF-IR vaccination, inhibit the growth of breast cancer via IFN-g secretion.
- IFN-g regulates tumor cell growth and survival via modulating IGF-IR signaling.
- IFN-g-induced SOCS1 mediates IGF-IR activity.
- IFN-g potentiates the association between SOCS1 and IGF-IR.
- IGF-IR-specific vaccine sensitizes tumors to Tamoxifen therapy.

REPORTABLE OUTCOMES

Abstract: Vaccination targeting IGF-IR sensitizes tumors to tamoxifen therapy in an anti-estrogen resistant mouse model.

CONCLUSION

We show that IFN-g secreting Th were primarily responsible for the observed tumor inhibition in mice after vaccination. The role of CD4⁺ T-cells in mediating cancer regression via biologic rather than direct immunologic mechanisms is increasingly being defined. IFN-g secreting Th contribute to the development of tumor "equilibrium",¹⁵ elicit chemokines which inhibit cell proliferation,¹⁶ and have recently been shown to stimulate stromal factors which induce cellular senescence and suppress angiogenesis.¹⁷ Our data suggests that a known negative regulator of IFN-g signaling, SOCS1,¹⁸ when induced by elevated levels of IFN-g interacting with IFN-gR on breast cancer cells, directly binds to IGF-IR and acutely inhibits receptor phosphorylation. This finding represents a novel mechanism for the anti-proliferative effects of IFN-g in cancer. It has been shown that IGF-IR phosphorylation leads to the subsequent activation of the downstream signaling cascades of phosphatidylinositol-3-kinase (PI3K/AKT) and Ras/Raf/MAPK.¹⁹ The activation of these pathways ultimately results in increased cancer proliferation and resistance to apoptosis. The AKT pathway has been implicated as part of a common signaling cascade associated with oncogene addiction to a variety of

observed in untreated mice. However, treatment with Tamoxifen in vaccinated tumor-bearing mice resulted in a further 38% reduction in growth compared to vaccinated, untreated animals ($p = 0.02$).

Aim 2.c. To determine whether IGF-IR vaccination induces diabetes or other toxicities in immunized mice.

Work on this sub-aim has started but there are no results yet to report.

Training Program.

My mentor and I have had intensive one-on-one meetings once every two weeks to evaluate the status of the project. I have attended our general laboratory research meetings every week where I have presented data four different times. In addition, I have attended a focused small group lab meeting on vaccine design and development every two weeks where I have presented data as well as given introductory lectures on new concepts within my scope of work that was unknown to the group.

proteins.¹ Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) antagonizes the PI3K/AKT pathway, regulating IGF-IR activity.²⁰ Inhibition of IGF-IR signaling by SOCS1 and resultant attenuation of AKT signaling represents the beginning of “oncogenic shock”; where proapoptotic signals become predominant and tumor growth is interrupted.²¹ During this time, the tumor may be more sensitive to a variety of anti-cancer therapies.²¹ Treating mice resistant to Tamoxifen therapy during vaccine induced oncogenic shock resulted in significant tumor inhibition.

There have been several reports of patients, with a variety of malignancies, achieving remarkable clinical response rates to standard chemotherapies administered directly after antigen-specific vaccination.^{22,23,24} In many cases the patients were retreated with drugs that they previously received and through which their disease progressed. These reports indicate that the immune response elicited through vaccination may have impacted the effectiveness of subsequent chemotherapy. Our data would suggest that vaccine-induced oncogenic shock played a role in sensitizing tumors to enhanced cell death with subsequent treatment in our model. The ability to more effectively treat cancer after immunization, or other forms of Type 1 cytokine inducing immune therapy, may contribute to the survival benefit observed after immunization in clinical trials of cancer vaccines.²⁵⁻²⁷

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APPENDICES

2012 American Association of Cancer Research Meeting Abstract:

Tamoxifen is a standard treatment for estrogen receptor (ER)-positive breast cancer patients. However, acquired or de novo resistance to therapy is a major clinical problem. In hormone-resistant tumors, there is increased activation of insulin-like growth factor-1 receptor (IGF-IR) and subsequent downstream signaling molecules, such as AKT. We have determined that IGF-IR is immunogenic in breast cancer and is a potential target for active immunization. It has been demonstrated that natural immunogenic human epitopes can be predicted by high binding affinity across multiple class II alleles, thus, we used a combined scoring system from five algorithms for predicting class II binding to determine Th epitopes of IGF-IR. Of the 20 potentially immunogenic peptides identified, five peptides (p1166-1181, p1212-1227, p1301-1316, 1307-1322 and p1311-1326) in the C-terminal domain were determined to elicit a predominantly inflammatory Th1 response compared to an immunosuppressive Th2 response in human PBMC. Overexpression of AKT induces tamoxifen resistance, while inhibition restores tamoxifen sensitivity. The tumor suppressor, PTEN, actively inhibits AKT. Data we have generated demonstrated that vaccination restored PTEN activity in tumor cells. We questioned if modulation of PTEN could render tumors in the anti-estrogen-resistant MMTV-neu mouse model

sensitive to tamoxifen therapy. Vaccination demonstrated a robust Th1 response ($p<0.001$) and concomitant inhibition of tumor growth by 85% compared to adjuvant only control animals ($p<0.001$). Treatment with tamoxifen in vaccinated tumor-bearing mice resulted in a further 38% reduction in growth ($p=0.02$). Thus, active immunization targeting IGF-IR may induce both immunologic and biologic effects resulting in the sensitization of the tumor to tamoxifen therapy.